LAB PROTOCOLS

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Nanodrop: DNA Concentrations

1. Ensure the program is selected to read “NUCLEIC ACID” where it says “Sample Type” (Top right of screen)

2. Load 2 µl of your blank onto the raised pedestal. (NOTE: this blank should be the solvent your DNA/RNA is suspended in. For example this could be EB buffer if you have just done a MiniPrep)

3. Lower the metal lever until it gently rests on the sample pedestal and click ‘BLANK’ to zero the spectrophotometer reading.

4. Using a tissue blot off the liquid from the pedestal and the contact on the underside of the lever.

5. Now load 2µl of your nucleic acid sample in the same manner as the blank and close the lever

6. Click ‘MEASURE’. After a short while it will display a concentration in ng/µl in the bottom right hand corner.

7. Also displayed is the UV/Vis spectrum. One should expect a clean ‘bell’-like curve that indicated a clean sample. If a rough and wavy spectrum is seen the sample is likely contaminated.

8. Blot off the sample from the contacts as before and repeat for other samples.

9. At the end of your use wash the contacts by adding 2µl water to the contacts and closing the lever a few times before blotting off the water to leave the machine dry.

Transformation into Chemically Competent Cells

Transforming Competent E. coli:

1. Thaw the DH5-alpha cells (from -80ºC freezer), plasmid sample (from -20ºC freezer), and the antibiotic stock (from -20ºC freezer) ON ICE.

2. Split the thawed cells into 2x 100µl aliquots.

3. To each aliquot add 1µl of plasmid DNA.

4. Incubate ON ICE for ~30 minutes. Incubation can be longer than this but certainly NO shorter.

5. During this incubation time prepare the antibiotic agar plates:

    a. Melt the agar jar for 15 minutes with the microwave on 'defrost'. Check on the bottle every 5 or so minutes to ensure it is not overflowing.

    b. Cool the agar bottle in 67ºC water bath. During this time switch on the the laminar flow hood to create a sterile environment.

    c. Once cool take the agar and petri dishes to the laminar flow hood.

    d. Add the appropriate volume of antibiotic to the agar bottle before pouring.

    e. Pour the agar evenly to no higher than the raised line. NOTE: place the lid resting on the edge of the dish so as to catch any drops that fall from the bottle.

    f. Leave the agar plates to set (~15-30 minutes)

6. Heat shock the bacteria by placing them in the 42ºC water bath for NO MORE THAN 45 SECONDS.

7. Immediately place the bacteria on ICE for 1 minute.

8. Add 800µl LB and incubate at 37ºC for 1 hour. NOTE: Place Eppendorfs in a Petri dish before placing in incubator.

9. Plate the bacteria under sterile conditions (bunsen burner ON):

    a. Place a glass spreader in ethanol.

    b. Flame the spreader to burn off the ethanol and let it cool.

c. Plate #1: spread 100µl of cell culture onto the plate

d. Plate #2: spin down the remaining cells from step 3. Discard the supernatant and re-suspend the pellet in 100µl of fresh culture. Spread this onto a second plate as in previous step

10. Incubate at 37ºC overnight.

Transforming Competent Cells - NEB alpha-5 cells

Follow the protocol as above with the following changes: If using the C2987 cells only use 50µl aliquots as supplied. Use 2µl of assembly product (1-5µl containing 1pg-100ng of plasmid DNA). In step 6, heat shock the bacteria by placing them in the water bath for EXACTLY 30 seconds. Leave on ice for 5 minutes. In step 8, add 950µl of SOC media and incubate as above.

NEB Q5 PCR

1. (Q5 is a high fidelity DNA polymerase supplied by New England Biolabs)

2. Calculate Tm and annealing temperatures of primer pairs using the NEB Tm Calculator.

3. *NOTE: If your primers are not entirely complementary to the template, remember to calculate annealing temperatures for the first annealing reaction (primer to template) not the whole primer length.*

4. Dilute each lyophilized primer (from IDT) with nuclease-free water to get solutions of 100 µM.

5. *e.g. if 80.8 nanomoles of primer, add 0.808 ml of nuclease-free water.*

6. Make up dilution in tube primer delivered in. Pipette up and down then spin gently in bench centrifuge to thoroughly mix. *NOTE: Do not contaminate water filter nozzle when collecting nuclease-free water.*

7. Add 45 µl of nuclease free water to a 1.5 ml Eppendorf. Add 5 µl of a primer to give a 1 in 10 dilution (10 µM). Repeat in a fresh Eppendorf for each primer. Mix thoroughly.

8. *NOTE: Change tip before replacing pipette into nuclease-free water to prevent contamination of all following samples.*

9. For a 25 µl PCR reaction add the following to PCR tubes on ice and pipette up and down to mix:

Template DNA	1 µl
10µM Forward Primer	1.25 µl
10µM Reverse Primer	1.25 µl
Q5® High-Fidelity 2X Master Mix (contains Q5 polymerase, Mg^{++}, buffer and dNTPs)	12.5 µl (giving final 1X concentration as recommended)
Nuclease Free water (making volume up to 25 µl)	9 µl

10. Take samples into Bayer/Berks lab to ProFlex PCR machine.

11. This system has a heated lid so there is no need to overlay sample with mineral oil before use.

12. Touch PCR machine control screen to check if all three blocks are currently in use. If not, use touch screen to set up PCR reaction programme. Set annealing temperature and melting temperatures as calculated earlier. Set extension time dependent on length of fragment: 30 seconds per 1000bp. Place PCR tube in appropriate block and close lid. Set programme running.

13. *NOTE: If there is a PCR tube in the block but the reaction programme has finished, remove the PCR tube and place it in the box in the top shelf of the fridge to the right of the PCR machine.*

14. When PCR reaction is complete remove the PCR tubes and do a DpnI Digest to remove template DNA.

DpnI Digest

DpnI is a restriction enzyme that cleaves methylated DNA. Treating PCR products with DpnI will therefore remove any template DNA derived from the bacteria. All the newly PCR-synthesised DNA will be unmethylated and thus unaffected.

1. Keeping the enzyme on ice, add 1 µl of DpnI to the PCR tube containing your sample. Gently invert a few times to mix contents.

2. Incubate at in a 37^{0}c water bath for 60 mins.

3. *NOTE: The enzyme does not need to be removed, as in a conventional restriction enzyme digest, as it will be denatured by the SDS in the loading dye (if running on gel) or by the transformation conditions (if transforming straight into bacteria).*

QIAquick Gel Extraction

The following method uses the 'QIAquick Gel Extraction Kit – Centrifuge Processing Kit' produced by QIAGEN. All centrifugation steps are at 17,900g/13,000rpm in bench centrifuge. Ensure ethanol has been added to the PE buffer and that QG buffer is yellow.

CAUTION – ALWAYS WEAR FACE SHIELD AND BUTTON UP LAB COAT FULLY WHEN WORKING AT THE UV LIGHT BOX

1. Weigh one Eppendorf per each sample to be extracted and label with sample name and Eppendorf mass.

2. Ensure gel has been stained following Staining DNA Gel protocol. Place ethidium bromide stained gel on the UV light box.

3. Ensure face shield is on before turning on UV light box at the wall and turning out main room light. *NOTE: Try to minimise the amount of time your DNA samples are exposed to the UV light.*

4. Use razor blade to cut desired band and place in labelled Eppendorf. *NOTE: Try to cut away as much excess gel as possible.*

5. Weigh each sample in Eppendorf and calculate mass of sample. Add 3 gel volumes of QG buffer to each sample. *e.g. Actual mass of gel sample = 0.2g, gel volume = 200 µl, therefore volume of QG buffer added = 600 µl*

6. Incubate in 50^{0}c water bath for 10 mins to dissolve the gel. Vortex every 2-3 minutes. *NOTE: Keep finger on lid of Eppendorf whilst vortexing.*

7. Check the mixture is yellow. If not, add 10 µl 3M Sodium Acetate and mix.

8. Add 1 gel volume of isopropanol and mix. If no isopropanol on the bench take a Duran bottle and collect some from the store in the cabinet underneath the fume hood.

9. Place one QIAquick spin column in a 2ml collection tube for each DNA sample. Label both the spin column and collection tube with sample name.

10. Pipette sample into centre of spin column and centrifuge for 1 min. Discard flow through and replace spin column in collection tube. The DNA should now be bound to the spin column membrane.

11. Add 0.5 ml of QG buffer to spin column and centrifuge for 1 min. Discard flow through and replace spin column in collection tube.

12. Add 0.75 ml of PE buffer to spin column and let stand for 2-5mins. Centrifuge for 1 min. Discard flow through and replace spin column in collection tube.

13. Centrifuge again for 1 min to remove residual buffer. Discard flow through and collection tube. *NOTE: Ethanol from the ethanol supplemented PE buffer can inhibit enzymes. To ensure all ethanol is removed from sample, you can stand spin column on cap on the bench to air dry for 5 mins.*

14. Place column in a clean, labelled, 1.5 ml Eppendorf tube.

15. For Gibson assembly applications a concentrated DNA sample is required, therefore add only 20 µl of EB buffer (elution buffer) to the centre of the spin column membrane. Allow to stand for 4 mins. Centrifuge for 1 min. DNA sample should now be in the flow-through collected in the Eppendorf.*NOTE: When placing spin columns in Eppendorf tubes into the centrifuge position the caps so that they either point in the opposite direction to the centrifuge rotates or down into the centre of the centrifuge.*

16. To maximise yield repeat steps 14 and 15 to make a second sample of DNA.

17. Use Nanodrop: Finding DNA Concentration protocol to determine DNA yield.

NOTE: this will not be an exact measurement as QG buffer interferes with UV/vis readings from the nucleic acid.

DNA Gel Electrophoresis

1. Make up agarose solution; need ~100ml for small gel or ~200ml for large gel. Percentage agarose needs to be appropriate for sizes of DNA fragments to be separated. Weigh agarose into weigh boat on balance. Pour into Duran bottle. Add appropriate volume of ½ x TBE buffer. *NOTE: After taking buffer, remember to turn bottle so tap is back over drip tray. NOTE: The higher the percentage of agarose, the less separation of larger fragments. e.g. separating ~0.9kb and ~5kb therefore making an 0.8% agarose gel = 0.8 g agarose in 100 ml ½ xTBE buffer.*

2. Tighten lid on Duran bottle and then release by ½ turn. Place in microwave on high for 2 minutes. Carefully tighten lid and remove from microwave by lid – CAUTION! VERY HOT! Ensure all the agarose has dissolved by swirling the bottle. *NOTE: Make sure lid is not so loose it will be pushed off as solution boils, but not too tight that pressure can't be released.*

3. Allow the solution to cool to 50-60^{0}c by placing in 55^{0}c water bath for ~30 mins. *NOTE: Alternatively, if using immediately, allow to cool slightly on bench before running under cold tap. Ensure the solution is moving inside the bottle to prevent localised setting of the gel against the glass.*

4. Tightly seal the ends of the gel tray either using rubber tubes or autoclave tape. Insert a comb with appropriate number of teeth for the desired number of wells.

5. Pour agarose gel into gel tray. Allow to set on the bench for ~15mins (small gel) or ~30mins (large gel). *NOTE: Ensure gel is close to, but not higher than the top of the comb indentations.*

6. When set, remove seal and place gel tray in DNA gel tank. Gently remove comb. Add enough ½ x TBE buffer to tank to just cover gel and fill all the wells. *NOTE: Do not remove tape before carrying the gel tray over to the electrophoresis bench – the gel is slippery and may slide out of tray if tipped. NOTE: TBE buffer crystallises when is dries. Rinse everything that has had TBE buffer on it before it dries.*

7. Add 5 µl of loading dye (New England BioLabs) to each DNA sample. Bench centrifuge for a few seconds to mix thoroughly. If samples are in PCR tubes place whole PCR tube in lidless Eppendorfs in order to centrifuge. *NOTE: Double check you are not accidentally adding the DNA ladder.*

8. Load 10 µl of DNA ladder (New England BioLabs) into first well. Gently load one DNA sample per well. Make a note of the contents of each well. *NOTE: Wells can take up to 30µl of sample, 15µl should be sufficient for extraction. Consider retaining some of sample in case mistakes are made alter in extraction procedure. NOTE: Load fragments of different expected sizes in neighbouring wells to make cutting bands out of the well for easier.*

9. Place lid on gel tank and connect leads up to 500V power pack. Ensure this is connected up the right way round so that the DNA will move in the right direction through the gel. Turn on power pack at wall and on unit. Press ‘Set’ and adjust voltage using dial to 100V, press ‘Set’ again. *NOTE: If then ‘Leakage’ light is on, there is too much buffer in the tank and it is short-circuiting the contacts. Turn off power pack, remove some buffer and re-start.*

10. After a 10 minutes check that dye is running in correct direction. Leave to run for 1 hour (small gel).

11. Turn off power pack on unit and on wall. Remove tank lid to remove gel. Replace lid if reusing tank. If not, pour away buffer and rinse tank.

Staining DNA Gels

1. Lift DNA gel tray out of 1/2 x TBE filled tank. Secure the gel with finger tips at the end of the tray and drain off the buffer.

CAUTION – ETHIDIUM BROMIDE IS CARCINOGENIC, ALWAYS WEAR GLOVES!

2. Carefully place in the gel in the ethidium bromide tray and replace lid. Turn on the shaker tray underneath. Rinse the gel tray. *NOTE: Lower the gel close to the surface of the ethidium bromide and let it slide off the tray to minimise splash.*

3. Leave for 20-30 minutes.

4. Remove the gel from the ethidium bromide using the large spatulas next to the tray. Drain off any excess ethidium bromide.

5. Carry the gel through to Transluminator, place inside and close the door. Zoom controlled by the computer and focus wheel manually adjusted at top of instrument. *NOTE: Try not to open the door to the instrument room with a gloved hand that is contaminated with ethidium bromide. Use the same hand to touch the gel and the other to use the computer. The computer is also contaminated with ethidium bromide so do not remove gloves to operate.*

6. Take picture and save in iGEM file.

7. Remove gel from Transluminator and wipe the instrument surface clean.*NOTE: If disposing of gel now, put in marked disposal bin by staining tray.*

Growing Liquid Cell Cultures

1. Switch on the lamina flow hood and carry out steps 2 and 3 in the hood.
2. Pipette 5ml LB broth into a 15ml Falcon tube.
3. Inoculate the Falcon tube with strain being grown using an inoculation loop.
4. Grow at appropriate temperature (37°C for E. coli and 30°C for Pseudomonas) with shaking overnight.

Frozen Cell Stock Preparation

1. Grow a liquid cell culture of the strain to be made competent overnight.

2. Pipette 0.666ml of liquid cell culture with 0.333ml of 60% glycerol (to give an overall percentage glycerol 20% and total volume of 1ml) into an eppendorf tube.

3. Put into the freezer at -80°C.

Streaking Plates

1. Obtain an LB agar plate.

2. Label the bottom of the plate.

3. Carry out the following steps in the lamina flow hood. Using a sterile inoculation loop touch the top of the bacterial stock that is to be plated out.

4. Gently spread the bacteria over the plate by streaking across one section of the plate. Then drag through the first section to streak over a second section of the plate. Then drag through the second section to streak the third section.

5. Incubate the plate overnight at the appropriate temperature (30°C for Pseudomonas and 37°C for E. coli).

Agar Plate Preparation

1. Put the agar (in a duran bottle) in the microwave on the ‘defrost’ setting for 20-30 minutes with the lid slightly unscrewed

2. Once there are no lumps in the agar, cool down in a 55 oC water bath (or, if needed quickly, under a cool tap, whilst swirling to avoid it setting.)

3. Add antibiotics as required to the duran bottle.

4. Label the bottom of the plates to be used.

5. Line the plates up at the back of the lamina flow hood. Take the lids of leaving them resting on the side of the plates. Fill the plates with the agar.

6. Allow the agar to set.

Electroporation of Pseudomonas

1. Keep everything on ice throughout the entire procedure. Prechill cuvettes on ice.

2. Transfer 6mL of an overnight culture into a Falcon tube. Centrifuge at 2000 rpm for 10 minutes and remove the supernatant.

3. Resuspend the pellet in 4mL sterile, 1mM HEPES solution (containig 10% glycerol by volume) and Centrifuge again for 5 minutes at 2000 rpm.

4. Discard the supernatant, resuspend the pellet in 4mL HEPES (with 10% glycerol) and repeat the centrifugation.

5. Remove the supernatant and resuspend the pellet in 400 uL HEPES (10 % glycerol). Store this on ice until use.

6. Add the DNA and transfer to an ice-cold cuvette.

7. Electroporate with a BIO-RAD electroporator. Make sure the time constant is equal to or greater than 4.

8. Immidiately mix the cells with 1mL SOC and transfer to a prechilled Eppendorf tube. Shake at 20°C for 2 hours.

9. Plate #1: Plate 100 uL on selective medium at a 1:10 dilution.

10. Plate #2: Spin the remainder at full speed for one minute, remove the supernatant and resuspend the pellet in 100mL of SOC and plate on a selective medium.

Reviving Freeze-Dried Cultures

1. Remove the freeze-dried strain from the cold room.

2. In the lamina flow hood, cut the top of the seal of the ampoule off with scissors.

3. Pipette 0.5 mL of the appropriate growth medium into the ampoule and mix by pipetting up and down, being careful to avoid frothing.

4. Leave on the bench for 40 minutes to resuspend.

Measuring Cell Density over Time

1. Open the top of the plate reader, and adjust the wires so they are set to read absorbance (use the guide in the machine).

2. Log on to the computer as lab admin.

3. Open the program Fluostar Optima.

4. Within the program, log on as lab user.

5. Click on test setup, then test protocol.

6. Click on the protocol called OD timecourse. Here you can adjust the number of cycles and the cycle time (time between each reading). You can also adjust the gain, which should be set to 750 for absorbance readings. The absorbance should be set to 595nm.

7. Click on the Concentration Volume Shaking tab. Here you can change the shaking settings so that the plate is shaken after each cycle, for the maximum time of 300s with a width of 5.

8. Click on the layout tab. Here you can change the settings to change the number of wells on the plate. The start value should be set to 1 and set to increase.

9. Click OK.

10. Click the reader configuration tab and ensure it is set to absorbance.

11. Click on measure, then temperature control. Here you can adjust the temperature to the correct temperature for the bacteria being used.

12. Click OK.

13. Click the open plate drawer button and insert your plate. Then click close plate draw.

14. Click measure, then click measure from this menu. Select your protocol. Here you can save the name of your reading.

15. Then click the start test run button.

16. By clicking the button with the graph icon you can monitor the progress.

17. Once measurements have finished being taken, click on the button that shows all readings the machine has taken.

18. Double click on your reading and copy and paste the results into a new excel file.

19. Save the file.

Miniprep: Plasmid Extraction

1. After growing cell cultures overnight in LB, resuspend pelleted cells in 250 µl Buffer P1 and transfer it into a microcentrifuge tube. Make sure that RNase A has been added to Buffer P1 before.

2. Add 250 µl of Buffer P2 and mix gently by inverting the tube 6 times. The cell suspension should turn blue. Try to carry on with the next step after less than 5 minutes to make sure that the lysis reaction doesn't proceed for too long.

3. Add 350 µl of Buffer N3 and mix the suspension immediately by inverting 6 times.

4. Centrifuge for 10 minutes at 13000 rpm (or 17900 x g). Apply the supernatants to a spin colums

5. Centrifuge again for 30-60 seconds and discard the flow-through.

6. Wash the spin column by adding 500 µl of the PB buffer and centrifuge for 30-60s.

7. Discard the flow-through and wash the spin column again by adding 750 µl of PE buffer. Centrifuge for 1 minute.

8. Discard the flow-through and centrifuge again for 1 minute. Now place the column in a 1.5ml microcentrifuge tube.

9. Add 50 µl of the EB buffer to the centre of the spin column. Let stand for 2 minutes and centrifuge for 1 minute.

Western Blot

1. Prepare the following solutions: SDS-sample buffer (0.05 M Tris-HCl pH 6.8, 10 % glycerol, 1 % SDS, 0.05 M DTT, 0.01% bromophenol blue); RAPID reducing buffer (20x solution: 62.8 g MOPS (free acid), 72.6 g Tris (base), 10 g SDS, 6.5 g Sodium Bisulfite, water to 500 ml) and cold blotting buffer (for 1 L: 3g Tris base, 14.4 g glycine, 200 ml methanol, 10 ml 10 % SDS, water to 1L, pH should be ~8.3)

2. After growing liquid cultures, spin down 4-5ml aliquots at 2000 rpm for 10 minutes. Remove the supernatant and resuspend the pellet in 100 uL of the SDS-sample buffer into an Eppendorf tube. Keep in the -20°C freezer for at least 30 minutes.

3. Boil the samples at 100°C for 10 minutes and spin for 1 minute at 13300 rpm. Store the samples at -20°C before use.

4. Run samples on Expedeon pre-cast gels. The 4-20 % gels are a good choice when blotting proteins of different sizes. The maximum sample volume for the 12 well gels is 35 µl and for the 17 well gels 20 µl. Load 10 µl of prestained kaleidoscope protein ladder in the first lane so that the orientation of the gel can be determined and the efficiency of blotting checked.

5. Run using 1 x RAPID reducing buffer. Gels should be run at 90 mA/gel with a maxium of 180 V; run time is 30 – 60 minutes.

6. Now, blot onto PVDF membrane using the BIORAD kit: Cut two sets each of three pieces of Whatman paper to the same size as the gel (stacking gel will be removed before blotting. Cut a piece of PVDF membrane to the size of the gel and immerse in methanol (use tweezers when handling the membrane when possible) for 5 minutes. Transfer the PVDF membrane to cold blotting buffer. Place a blotting cassette black side down in a large box of cold blotting buffer. Immerse a blotting pad and one of the sets of Whatman paper in the buffer. Place the blotting pad and then the Whatman paper onto the cassette smoothing out any bubbles with a spreader.

7. Remove the gel from the plastic casing, place in blotting buffer and then onto the cassette. Add the PVDF membrane to the cassette making sure there are no bubbles. Immerse a blotting pad and the second set of Whatman paper in the buffer. Place the Whatman paper and then the blotting pad onto the cassette smoothing out any bubbles with a spreader. Close the blotting module and place in the running tank. If only one gel is to be blotted then fill the other space with a second cassette containing two blotting pads. Insert a cooling block containing frozen Milli-Q. Fill tank with blotting buffer, place tank in an ice bucket and run at 0.5 A for 1 hour. Disassemble blotting apparatus and place blot in 5% milk powder in PBS with shaking overnight.

8. The next day, tinse the blot in PBS and then wash in fresh PBS with shaking whilst preparing primary antibodies.

9. Dilute primary antibody in 1% milk powder in PBS according to the table at the end of the protocol (serial dilutions are usually necessary in order to get a sensible dilution factor). 10 ml is required per blot. Incubate at room temperature with shaking in a clean square petri dish for at least one hour – seal the petri dish with parafilm to ensure that the blot does not dry out.

10. Rinse the blot with PBS and then wash (timings are minimum, not exact, use tweezers when moving blot): 10 mins PBS; 10 mins PBS with 0.2 % Tween 20; 10 mins PBS with 0.2 % Tween; 10 mins PBS

11. Dilute the secondary antibody in 1 % Marvel in PBS (1 in 20000 is a good starting point, if the background is too high then increase to 1 in 40000). 10 ml is required per blot. Incubate at room temperature with shaking for at least 1 hour.

12. Rinse the blot again with PBS and then wash (timings are minimum, not exact): 10 mins PBS; 10 mins PBS with 0.2 % Tween 20; 10 mins PBS with 0.2 % Tween; 10 mins PBS

13. In order to detect the proteins by chemiluminsecence, prepare the HRP (horseradish-peroxidase) substrate. For a 7 x 8.5 cm gel use 3 ml luminal reagent + 3 ml peroxide solution. For a 10 x 10 cm gel use 5 ml luminal reagent + 5 ml peroxide solution. Allow the substrate to reach room temperature. Protection from light is not required

14. Using tweezers, lift the blot from the petri dish and then touch the edge of the blot on some blue roll to remove excess liquid. Place on a piece of OHP film. Pour the HRP substrate onto the blot and incubate at room temperature for 5 minutes.

15. Lift the blot from the substrate using tweezers and touch the edge of the blot on some blue roll to remove excess liquid. Cover the blot with OHP film and remove any air bubbles.

16. Place the blot on the gel doc and focus using white light.

17. Now, make sure that the doc is set to no light and no filter. Set up multiple exposure runs each of 30s for at least 30 minutes (i.e. 60 exposures). Make sure the software is set to sum the images (you will get 60 images, each with effectively a longer exposure time). Save the best image for analysis.

Restriction Digest

1. For a double digest, make sure that both enzymes are compatible and that a buffer exists in which both of them can work.

2. Add 6 uL of water into an empty Eppendorf tube.

3. Add 1uL of the buffer and 2 uL of the DNA or plasmid.

4. Add 0.5uL of each enzyme solution. Make sure the restriction enzymes are kept on ice before use.

5. Incubate for 1-2h at 37°C.